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CONTROLLABLE GENE THERAPY: RECENT ADVANCES IN NON-VIRAL GENE DELIVERY

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INTRODUCTION TO GENE THERAPY APPROACHES

Many diseases have become a target for somatic gene therapy, including acquired, multifactorial diseases such as cancers, arthritis and AIDS as well as genetic disorders such as cystic fibrosis and Duchenne muscular dystrophy (Anderson, 1992; Ledley, 1993a, b; Ledley, 1994a, b; Anderson, 1995; Hodgson, 1995). Gene therapy methods have been designed to introduce genetic information into a patient's cells to enable these cells to produce beneficial proteins to correct or modulate a disease. Different biological targets and pathologies will require different gene therapy methods. These methods are intended to overcome some limitations associated with the clinical use of protein drugs, including low bioavailability, poor pharmacokinetics or cost of manufacture.

Over the past few years, rapid progress in mapping of the human genome associated with major advances in controlling the delivery and expression of therapeutic genes in vivo has enhanced the promise of gene therapy. Several methods for transferring genes to a patient's cell have been explored over the last decade (Miller, 1992; Kay et al., 1993; Culver and Blease, 1994; Glorioso et al., 1994; Ledley, 1995; Rolland and Tomlinson, 1996). Each of these methods offers distinct clinical opportunities and risks as well as potentials for commercialization. They include the use of: (*i*) cells, genetically modified ex vivo with viruses or other gene-transfer methods prior to their re-introduction into the patient's body, (*ii*) modified viruses, based for instance on replication-defective retroviruses, adenoviruses and adeno-associated viruses, and (*iii*) DNA plasmids, formulated using synthetic delivery systems for direct in vivo administration.

Early approaches used modified viruses or implanted cells to transfer genes into the body. Since the first clinical trial in gene therapy in 1990 (Culver and Blaese, 1994), involving an ex vivo approach in which retroviral vectors were used to introduce the adenosine deaminase gene into the white blood cells of patients suffering from severe combined immunodeficiency (SCID), there has been a lot of excitement and hope about the potential of these approaches for treating a broad variety of diseases. As a consequence, most of the U.S. RAC (Recombinant DNA Advisory Committee)-approved clinical trials (involving about 600 patients in more than a 100 clinical studies) are based to date on cell- and viral-mediated approaches. However, despite the promise of the first gene therapy clinical trial, concerns have been raised about the safety and efficacy of cell- and viral-mediated approaches for the treatment of other diseases, including cystic fibrosis, muscular dystrophy and familial hypercholesterolemia (Marshall, 1995). A recent ex vivo gene therapy clinical trial for muscular dystrophy failed to show improvement in muscle strength of the boys afflicted with the genetic disorder (Mendell et al., 1995). Similarly, a complex

Targeting of Drugs 5: Strategies for Oligonucleotide and Gene Delivery in Therapy
Edited by Gregoriadis and McCormack, Plenum Press, New York, 1996

and invasive procedure in five patients with familial hypercholesterolemia, involving partial hepatectomy, stable transduction of isolated hepatocytes with the gene encoding the low density lipoprotein receptor and transplantation of the autologous, modified hepatocytes via portal vein injection, failed to produce significant clinical benefit (Williams, 1995).

Other recent clinical data in cystic fibrosis patients treated with adenoviral vector containing the gene coding for the cystic fibrosis transmembrane conductance regulator protein (CFTR) have also been disappointing (Knowles et al., 1995). Due to their natural ability to infect cells efficiently, several viruses, such as retrovirus, adenovirus and adeno-associated virus, had been investigated for in vivo viral-mediated gene delivery (Miller, 1992; Rosefeld et al., 1992; Kay et al., 1993; Glorioso et al., 1994). Each of these has different biological properties. Retroviral vectors can introduce genes permanently into somatic cells by integration into patient's chromosomal DNA. Retroviruses only infect replicating cells. Thus, the resultant permanent integration of therapeutic genes minimizes the ability of the physician to modify or terminate the therapy in response to any adverse side-effects or cure of the disease. Besides, the permanent integration of genes into host chromosomes may result in activation of oncogenes or inactivation of tumor-suppressor genes. Adenoviruses efficiently infect non-dividing cells and do not integrate genes into the host genome. However, safety concerns have been raised in clinical trials about the immune and inflammatory responses triggered by adenoviral vectors. These events would be a severe limitation in the repeated administration of genes using adenoviral vectors. In addition, even though viruses are designed to be replication-defective, there is a potential risk of generating an infectious, replication-competent virus during the production or use of viral vectors for gene transfer.

The direct in vivo administration of genes to patients using non-viral technologies, to cause the controlled production and distribution of therapeutic proteins within the body, would represent an ideal approach for clinical practice. Plasmid-based gene medicines are designed to control the location and function of administered therapeutic genes within the patient's body (Ledley, 1995; Tomlinson and Rolland, 1996; Rolland and Tomlinson, 1996). Gene medicines, that consist of both a DNA plasmid-based gene expression system containing a therapeutic gene and a synthetic gene delivery system, represent a potentially safe and effective gene therapy method for the treatment of a wide variety of acquired and genetic diseases. The successful development of plasmid-based gene medicines will also require a cost-effective, robust and reproducible manufacturing process. These bio-pharmaceutical products will have to be stable upon storage, preferably as a single vial preparation.

PLASMID-BASED GENE MEDICINES

Gene medicines are designed to be administered to a patient by conventional routes using convenient methods such as direct injection into target tissue (e.g., muscle, tumors), inhalation or intravenous injection. These semi-synthetic products are intended to have low toxicity due to the use of synthetic material for gene delivery and non-integrating plasmids. Plasmids degrade within the body, leading to a finite duration of gene expression. At the doses applied, plasmids do not appear to integrate into host chromosomes. Thus, they would neither activate oncogenes nor inactivate tumor suppressor genes. Such favorable properties of gene medicines may enable a physician to control gene-dosing regimens according to therapeutic needs.

Modern advanced drug delivery, that includes a better understanding of (patho)-physiology, cell and molecular biology, teaches that each biological target for gene therapy would require a specific gene delivery and expression system. The development of target-specific non-viral gene therapies requires the combination of a synthetic gene delivery system designed to deliver the therapeutic gene to a specific target cell and a gene expression system that controls gene function within each target cell.

Gene delivery systems are designed to control the location of a gene within the body *'DART'* by effecting the distribution *(D)* and access *(A)* of the gene expression system to the target cell, and/or recognition *(R)* by a cell-surface receptor followed by intracellular trafficking and nuclear translocation *(T)* . A synthetic gene delivery system should serve both to protect a gene expression system from premature degradation in the extracellular milieu and to effect adequate non-specific or cell-specific delivery to a target cell. Other elements in a gene delivery system may facilitate the intracellular trafficking of a gene

expression system (Table 1).

Plasmid-based gene expression systems contain a therapeutic gene and other DNA sequences to control the in vivo production of a protein *'ACT'*, i.e., gene expression and protein secretion. They may contain genetic sequences that control the amount *(A)* of transcripts and consequently produced protein, the cell- *(C)*, or eventually the disease-, specific activation of gene expression and the timing *(T)* of protein production. Such sequences may include cell-specific promoters and enhancers to cause the expression of the therapeutic gene to be restricted to specific sites within the body (Sellheyer et al., 1993; Coleman et al., 1994). They may also contain transcript stabilizers that increase the chemical stability of transcribed messenger RNA and consequently the level and duration of production of a gene product (Schwartz et al., 1994). Persistence elements may need to be incorporated into some gene expression systems to prolong the production of therapeutic proteins in specific tissues. Additional genetic sequences can be used to control the secretion of a gene product from the cells. Gene switches can also be introduced in a gene expression system to enable the function of an administered gene to be activated using low molecular weight drugs (Wang et al., 1994). These can be designed to be cell-specific. Such gene-switches would enable the physician to control expression of a therapeutic gene in the patient's body, by turning it on and off with specific drug molecules.

Table 1: Control of gene location and function by plasmid-based gene medicines

Gene Delivery System 'DART'	Gene Expression System 'ACT'
Distribution	Amount
dispersion	efficient transcription
retention	RNA processing and stabilization
protection	drug control
Access	Cell
condensation	cell-specific
non-specific uptake	disease-specific
Recognition	Timing
cell-specific targeting	drug control
receptor-mediated uptake	episomal replication
Trafficking	
endosomal release	
nuclear localization	

Gene medicines can be considered as in vivo protein production platforms for a variety of therapeutic proteins, including those having an autocrine effect (e.g., LDL receptor), a paracrine effect (e.g., insulin-like growth factor 1) or an endocrine effect (e.g., factor VIII).

A fundamental challenge for the effective delivery of plasmid-based gene expression systems to a target tissue is the control of the surface and colloidal properties of plasmids in a biological environment. Plasmids are colloidal systems of varying size, as determined by several experimental techniques including dynamic light scattering. For example, plasmids of less than 10,000 base pairs have a mean size <200 nanometers (Figure 1). Plasmids are also very hydrophilic molecules. They have a highly negative surface charge. It is apparent that the colloidal and surface properties of plasmids determine their biological distribution, cellular uptake, and intracellular trafficking and nuclear translocation (Ledley and Ledley, 1994; Lew et al., 1995; Liu et al., 1995; Thierry et al., 1995; Zabner et al., 1995). Due to their colloidal nature, hydrophilicity and highly negative surface charge, plasmids do not efficiently cross intact biological barriers such as continuous endothelium, keratinized epithelium, mucosal epithelium or blood-brain barrier. In addition, the penetration of DNA plasmids into cells without transient permeabilization of the plasma membrane and/or disruption of the endosomal membrane, represents a very ineffici nt

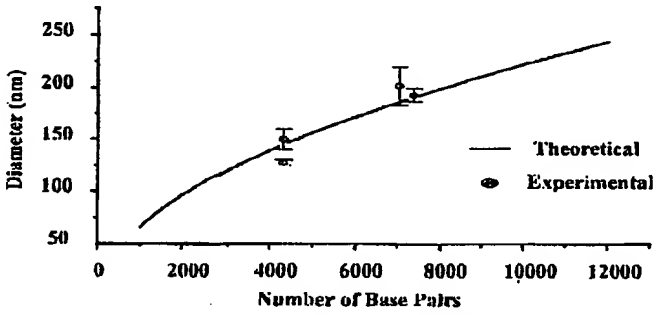

Fig. 1. Hydrodynamic diameter of DNA plasmids with different molecular weight estimated by dynamic light scattering (theoretical computation based on the Porod-Kratky model, with a DNA persistence length of 50 nm).

process. Their diffusion through extracellular matrices, such as the connective tissue in skeletal muscle, is also very limited.

It is postulated that gene delivery systems that control the chemical and biological stability of plasmids as well as their colloidal and surface properties can lead to enhanced delivery of plasmids to cells in vivo. The application of advanced drug delivery principles and technology to gene therapy provides a basis for the development of non-viral gene therapies that are being examined in the clinic at an increasing rate.

This contribution describes the development of several non-viral technologies, with an emphasis on the control of the location of administered genes in several tissues, including skeletal muscle, pulmonary vascular endothelium and hepatocytes, after direct intramuscular administration, inhalation and intravenous injection, respectively. The design and development of synthetic gene delivery systems that effect the in vivo distribution, access, recognition and intracellular trafficking '*DART*' of a gene expression system is exemplified. Control of the 'distribution', i.e., protection, dispersion and retention of a gene expression system in a target tissue is illustrated by the use of interactive polymeric gene delivery systems that enhance the delivery of genes to muscle cells after their direct intramuscular administration. Modulation of the 'access' of gene expression systems to a target tissue is exemplified by the use of self-assembling systems based on cationic polar lipids. The characterization of the resulting particulate gene medicines is reported, as is the influence of their physicochemical properties on the efficiency of gene delivery. This contribution also describes approaches to effect the 'recognition' of targeted gene delivery systems by specific cells using targeting ligands bound to cationic carriers, in particular glycopeptides. The intracellular 'trafficking' of a gene expression system to the nucleus of a target cell, in particular by using synthetic amphipathic peptides to control the endosomal release of plasmids is also reported.

CONTROL OF PLASMID DISTRIBUTION IN MUSCLE

A few years ago, the direct injection of DNA plasmids in isotonic saline into muscle had been found to result in the uptake and gene expression in muscle cells (Wolff et al.,1990, 1991, 1992a, b; Acsadi et al., 1991; Manthorpe et al., 1993). However, the intramuscular administration of unformulated plasmids (so-called 'naked DNA') results in low levels of gene uptake and expression (Jiao et al., 1992; Ulmer et al., 1993; Davis et al., 1993a, b; Raz et al., 1993; Levy et al., 1994). Only a very small fraction of the injected plasmid is taken up by a small number of muscle cells (< 1%). The cellular uptake of plasmids from an isotonic saline formulation is a saturable process that results in very low and highly variable levels of in vivo production of therapeutic proteins.

We have designed and developed gene delivery systems that control the distribution of gene expression systems in muscle tissue after their direct intramuscular administration and consequently enhance their cellular bioavailability (Mumper et al., 1995b; 1996). Such systems result in a significant increase in the steady-state levels of both reporter and

therapeutic genes expressed in muscle as compared to gene expression systems injected in isotonic saline. These synthetic gene delivery systems result in high and reproducible levels of gene expression in muscle for several weeks. They provide opportunities, for example, for the treatment of both muscle and peripheral nerve disorders as well as for the sustained production and systemic secretion of therapeutic proteins and antigens.

These interactive colloidal polymers include polyvinyl derivatives that interact with a DNA plasmid via hydrogen bonding and hydrophobic interactions. We have demonstrated that polyvinyl pyrrolidone and polyvinyl alcohol interact with plasmids through hydrogen bonding. Dynamic dialysis, FT-IR and microtitration calorimetry studies have been used to characterize the interaction between polyvinyl derivatives and plasmids (Mumper et al., 1995b; 1996). This interaction results in a significant protection of plasmids from nucleases, probably by providing an hydrophobic coating of the plasmid. Polyvinyl pyrrolidone-based formulations are hyperosmotic and result in an improved dispersion of plasmids through the extracellular matrix of the muscle tissue, most likely by increasing intercellular spacing. These polymers may also facilitate the uptake of plasmids by muscle cells by increasing their hydrophobicity and reducing their net negative surface charge. Immunohistochemical staining of rat muscle sections after the intramuscular injection of either 'naked DNA' or a polyvinyl pyrrolidone-based formulation of plasmid shows that the polymeric gene delivery system significantly increases the number, as well as the distribution, of cells expressing a β-galactosidase gene (Mumper et al., 1995b; 1996). The improved tissue dispersion and cellular uptake of plasmids using interactive polymers can lead to pharmacological levels of proteins after a single intramuscular injection. Figure 2 shows the expression of chloramphenicol acetyl transferase in muscle after the administration of plasmids in various formulations of isotonic polyvinyl pyrrolidone. The study demonstrates that maximal gene expression in rat tibialis muscle at 7 days occurs using the 5% (w/v) polymer-based system. Up to a 10-fold enhancement of gene expression over 'naked DNA' is observed (Figure 2). In addition, intramuscular injection of polyvinyl pyrrolidone-based formulations of plasmid results in highly reproducible levels of gene expression in contrast to the variable levels generally observed with 'naked DNA'.

CONTROL OF PLASMID ACCESS TO LUNGS

A number of approaches have been proposed to control the access of plasmids to a target cell and enhance their cellular uptake. These include the use of a number of different types of condensing carriers, such as cationic lipids, charged synthetic polymers and peptides that act in a non-specific manner (Felgner et al., 1987; Felgner and Ringold, 1989; Tomalia et al., 1990; Nabel et al., 1990, 1992; Zhu et al., 1993; Haensler and Szoka, 1993; Staedel et al., 1994; Boussif et al., 1995; Mumper et al., 1995a). Lipid-based gene delivery systems have been widely used for in vitro and in vivo preclinical studies as well as in recent clinical trials (Ono et al., 1990; Yoshimura et al., 1992; Nabel et al., 1992, 1993; Philip et al., 1993; Alton et al., 1993; Canonico et al., 1994; Conary et al., 1994; Caplen et al., 1994; Thierry et al., 1995; Liu et al., 1995; Lesoon-Wood et al., 1995).

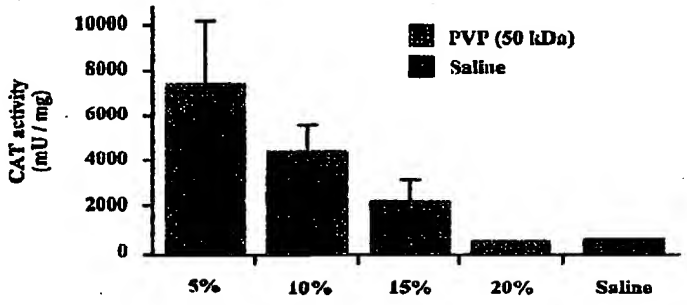

Fig. 2. Expression of chloramphenicol acetyl transferase in rat tibialis muscle 7 days after a single injection of 50 μg of plasmids in isotonic saline or formulated with polyvinyl pyrrolidone at various concentrations in isotonic saline (n = 10).

Although (pH-sensitive) liposomes, immunoliposomes and proteoliposomes are able to mediate the transfer of encapsulated gene expression systems into the target cells (Nic lau et al., 1983; Wang and Huang, 1987, 1989; Gould-Fogerite et al., 1989; Nicolau and Cudd, 1989; Legendre and Szoka, 1992; Alino et al., 1994; Von Der Leyen et al., 1995; Baru et al., 1995), the levels of expression that are generally achieved remain low . In addition, the encapsulation of gene expression systems into liposomes has several limitations, such as low yield of encapsulation and requirement for the separation of free from encapsulated plasmid. The encapsulation procedure may also result in degradation or alteration in the structure of the gene expression system.

As an alternative, cationic lipids have been used to deliver gene expression systems to a variety of tissues in vivo. They reduce the net negative surface charge of plasmid-based gene expression systems, resulting in condensation of plasmids into discrete nanoparticles with defined colloidal properties. Such lipids can form stable complexes with gene expression systems (Felgner and Ringold, 1989; Gao and Huang, 1991; Felgner et al., 1994; McLachlan et al., 1994; Stuedel et al., 1994). The neutralization of the negative surface charge of plasmids is also intended to reduce charge-charge repulsion at the surface of biological membranes, thus enhancing plasmid access into the target cells. However, to enable the plasmid to be released from the endosomes following the endocytic uptake of the plasmid/lipid complex by a cell, additional lipids that can fuse with endosomal membranes may be required. Recent data suggest that plasmid/lipid complexes also need to dissociate, probably in the endosomes, to allow the plasmid to translocate to the nucleus of the target cell by a mechanism still not elucidated (Zabner et al., 1995).

Plasmid/cationic lipid complexes can be prepared as such to have defined physicochemical properties (size, shape, surface characteristics) (Rolland et al., 1994; Gong et al., 1994). Cationic lipids such as DOTMA (dioleyloxypropyltrimethylammonium chloride), the first cationic amphiphile specifically designed for gene transfer, have been extensively used in vitro and in vivo for gene delivery (Felgner et al., 1987; Felgner and Ringold, 1989; Brigham et al., 1989; Nabel et al., 1990; Stribling et al., 1992; Zhu et al., 1993). Since the characteristics of a plasmid/lipid complex may both control the biological stability and distribution of plasmid-based gene expression systems as well as enhance cellular uptake by non-specific adsorptive mechanisms, the physicochemical properties of DOTMA-based gene delivery systems have been evaluated (Rolland et al., 1994; Gong et al., 1994; Tomlinson and Rolland, 1996). Table 2 presents some of the colloidal and surface properties of DOTMA-based systems and their transfection efficiency in vitro in different cell lines.

As illustrated in Table 2, the physicochemical properties of plasmid/lipid complex s can be controlled by adjusting, for example, the ratio of cationic lipid to plasmid. The mean diameter of the resulting nanoparticles, as determined by dynamic light scattering, further depends on the concentration of formulated plasmid, the method of preparation and the chemical structure of the cationic lipid. The surface charge of a plasmid/lipid complex, determined by Doppler electrophoretic light scattering and expressed as the zeta potential of the particulate system, can also be controlled by the ratio of lipid to plasmid. For instance, the zeta potential changes from a negative to a positive value, with a concomitant

Table 2: Characterization of DOTMA-based gene delivery systems

Lipid composition DOTMA:co-lipid (mol %)	Lipid: plasmid ratio (+/-)	Mean Diameter (nm)	Zeta potential (mV)	Transfection efficiency in vitro a	b
DOPE (55:45)	0.5	173	- 27.2	109±59	0*
DOPE (55:45)	0.8	217	- 26.7	177±73	0*
DOPE (55:45)	2	218	+ 21.9	0*	70±47
DOPE (55:45)	3	182	+ 51.6	0*	142±12

* below detection limit

Expression levels of CAT (pg/ mg protein) 48 h after transfection of (a) BEAS 2B lung epithelial cells and (b) HIG 82 rabbit synoviocytes, in presence of 10% serum (2 µg plasmid; 24-well plate; cell density: 100,000 ; CAT assay: ELISA, Boehringer Mannheim)

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Transfection efficiency in vitro	
a	b
109±59	0*
177±73	0*
0*	70±47
0*	142±12

) BEAS 2B lung
serum (2 µg
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enhanc ment in complexation efficiency as estimated by gel electrophoresis, by increasing the cationic lipid to plasmid ratio (Table 2). The transfection efficiency observed in different cell lines is dependent on the net charge of the complex. However, the optimal composition of the lipid-based system to achieve maximal in vitro gene expression varies from cell line to cell line (Table 2). In addition, there appears to be no systematic correlation between in vitro transfection efficiency and in vivo gene expression. Additional studies will be required to enable in vitro characterization assays to be used to predict the in vivo efficiency of lipid-based gene delivery systems.

The lung appears to respond well to lipid-mediated gene transfer. Cationic lipids have been used over recent years to deliver genes to the lung of various species, including mice, rats, and sheep by either direct aerosol delivery or systemic administration (Brigham et al., 1989, 1993; Hazinski et al., 1991; Debs et al., 1992; Stribling et al., 1992; Bout et al., 1993; Canonico et al., 1994). Successful expression of α1-antitrypsin (AAT) and CFTR in the lung in preclinical studies has led to clinical trials for gene therapy of the genetic deficiencies of these proteins, i.e., AAT insufficiency and cystic fibrosis (Yoshimura et al., 1992; Alton et al., 1993; Hyde et al., 1993; Canonico et al., 1994; Logan et al., 1995).

Other studies have also shown that DOTMA-based delivery systems with defined colloidal and surface properties were able to achieve pharmacological effect after intravenous injection of a gene encoding the enzyme prostaglandin G/H synthase (P G/H synthase) in animal models of pulmonary inflammation (Conary et al., 1994; Brigham et al., 1994). A single intravenous injection of a plasmid encoding the P G/H synthase formulated with a DOTMA-based gene delivery system protected rabbits against endotoxin-induced pulmonary hypertension. The enhanced access of plasmid/lipid complexes to the lung endothelium after intravenous administration may result from the control of the colloidal and surface properties of the particles (hydrophobicity and zeta potential). These defined properties may facilitate the ionic and/or hydrophobic interaction of the particles with the lung vascular endothelium and this is probably aided by the fact that the pulmonary vasculature is the initial capillary bed encountered after intravenous injection.

CONTROL OF PLASMID RECOGNITION BY HEPATOCYTES

The cell-specific delivery of gene expression systems involves the use of targeting ligands that recognize cell-surface receptors involved in receptor-mediated cell entry. A variety of targeting ligands, such as asialoglycoprotein, transferrin, lung surfactant proteins, insulin, folic acid and carbohydrates have been conjugated to polypeptides (primarily poly-L-lysine (PLL)) which condense plasmid-based gene expression systems through ionic interactions (Wu et al., 1991; Wagner et al., 1991; Wilson et al., 1992; Midoux et al., 1993; Gottschalk et al., 1994; Perales et al., 1994a, b; Baatz et al., 1994; Ross et al., 1995; Erbacher et al., 1995; Ding et al., 1995; Ferkol et al., 1996).

Asialoorosomucoid-PLL/plasmid complexes have been used, for example, to establish hepatic expression of reporter genes in normal animals (Wu and Wu, 1988; Chowdhury et al., 1993), low-density lipoprotein (LDL) receptor in LDL-deficient rabbits (Wilson et al., 1992), albumin in analbuminemic rats (Wu et al., 1991) and methylmalonyl CoA mutase in mice (Stankovics et al., 1994). Evidence of specific gene delivery to hepatocytes in vivo has been obtained with the use of hepatocyte-specific promoters (Wu et al., 1989) and histological analysis (Chowdhury et al., 1993). The delivery of plasmids to hepatocytes in vivo has also been reported using poly-L-lysine covalently coupled to galactosyl residues as targeting ligands (Wu et al., 1991; Wilson et al., 1992; Wu and Wu, 1993; Stankovics et al., 1994; Frese et al., 1994; Perales et al., 1994a, b). These approaches, though successful in obtaining expression of therapeutic genes in animal models, present several pitfalls associated with the use of poly-L-lysine which is toxic and has variable quality. The use of asialoglycoprotein as a targeting ligand also presents the risk of inducing an immune response.

Since the current methods used for producing plasmid-based nanoparticles for hepatocyte targeting have limitations, we are developing synthetic glycopeptide-based gene delivery systems for the in vivo transfer of gene expression systems to hepatocytes. The design of such hepatocyte-specific gene delivery system is based on knowledge of the structure and function of the liver and principles of cellular uptake and intracellular trafficking of plasmids. The gene delivery system comprises: (i) a galactosylated peptide that both condenses the plasmid and enables specific recognition and binding to asialo-

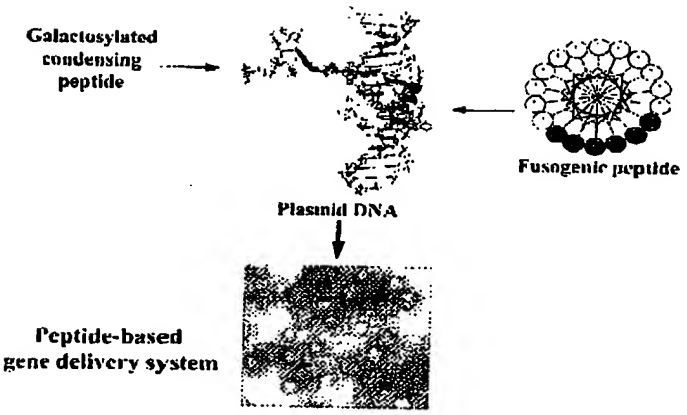

Fig. 3. Elements of a glycopeptide-based system for receptor-mediated gene delivery. The transmission electron micrograph shows the toroidal nanoparticles (~ 100 nm) obtained by self-assembly of plasmids with a galactosylated condensing peptide and a pH-sensitive fusogenic peptide (GM225.1).

glycoprotein receptors, and (ii) an amphipathic, pH-sensitive peptide that enables the plasmid to leave the endosomes prior to their fusion with lysosomes and to enter the cytoplasm (Figure 3).

The galactosylated peptides have been prepared using an automated solid-phase synthesis. These glycopeptides are based on novel synthetic cationic peptides (~10 amino acids) that condense plasmids into monodisperse nanoparticles of a diameter below 100 nanometers (Gottschalk et al., 1996; Tomlinson and Rolland, 1996). They are covalently linked to a spacer with no secondary structure that gives optimal distance for interaction of the terminal galactosyl ligands of the condensed plasmid with the asialoglycoprotein receptor (Tung et al., unpublished data). Mono-, bi-, tri- and tetra-antennary galactosyl-condensing peptide conjugates have been examined, since the number and clustering of terminal galactosyl residues have been shown to control the affinity between these ligands and the asialoglycoprotein receptor (Lee et al., 1983; Monsigny et al., 1994). The specific and enhanced delivery of a reporter gene to cells presenting the asialoglycoprotein receptor (HepG2 cells) using a galactopeptide-based gene delivery system has been observed (Mumper and Wadhwa, personal communication). As compared to a non-specific peptide-based gene delivery system (without targeting ligand), the hepatocyte-specific galacto-peptide-based gene delivery system increased significantly the levels of reporter gene expression. The receptor-mediated endocytic uptake of the system resulted in a higher magnitude of gene expression for a neutral complex, since for a positively charged one the non-specific uptake of the nanoparticles probably dominated due to the ionic interactions with the plasma membrane. These galactosylated peptides are currently being investigated in vivo for their targeting and gene transfer capabilities.

The key challenge for effective hepatocyte non-viral gene therapy is to produce colloidally stable plasmids with a mean diameter below about 100 nanometers that give persistent high levels of expression. This size enables penetration of plasmid complexes through the sinusoidal endothelial barrier of the liver (which has gaps of between 100 nm and 200 nm and no basement membrane (Wisse et al., 1984) and then into the space of Disse. The plasmid complexes need then to bind to specific hepatocyte receptors, such as the asialoglycoprotein receptor, to induce receptor-mediated endocytosis. Thereafter, for an efficient plasmid translocation to the hepatocyte nucleus, the delivery system may need to

contain a pH-sensitive lytic agent to facilitate the release of the plasmid from th endosomes into the cytoplasm as exemplified in the next section.

CONTROL OF PLASMID INTRACELLULAR TRAFFICKING

A current limitation for efficient gene delivery via both non-specific and receptor-mediated gene transfer is an effective exit of a gene expression system from the endosomal compartment prior to the fusion of endosomes with lysosomes. The ability of viruses to release their viral genome from endosomes, by either disruption or fusion with endosomal membranes, was first explored as a means to improve the intracellular trafficking of plasmids. The association of replication-defective adenoviral particles to complexes, such as transferrin-PLL/plasmid (Curiel et al., 1991, 1992; Cotten et al., 1992; Wagner et al., 1992; Harris et al., 1993), folate-PLL/plasmid (Gottschalk et al., 1994), or asialo-glycoprotein-PLL/plasmid (Cristiano et al., 1993; Wu et al., 1994), for receptor-mediated gene delivery resulted in a significant increase in transfection efficiency in vitro. Endosomal lysis is mediated by the penton protein on the surface of the virus that undergoes a change in tertiary structure upon acidification of the endosomal compartment (Seth, 1994). This conformational change results in partitioning of the viral protein in the endosomal membrane which causes release of the endosomal content in the cytoplasm (Prchla et al., 1995). Peptides derived from the N-terminal sequence of the influenza virus envelope glycoprotein have also been used to induce membrane fusion at acidic pH. The addition of the peptides to transferrin-PLL/plasmid complexes resulted in an increased transfection efficiency in vitro (Wagner et al., 1992b; Plank et al., 1994).

Other methods for enhancing the release of plasmids into the cell cytoplasm from endosomes, following both non-specific and receptor-mediated gene delivery, have been described using synthetic compounds. Lysosomotrophic agents such as chloroquine, monensin, ammonium chloride and Brefeldin A have been shown, for example, to enhance in vitro gene transfer (Plank et al., 1992; Cotten et al., 1993; Wadhwa et al., 1995). Haensler and Szoka have successfully enhanced gene transfer in vitro using a synthetic amphipathic peptide (GALA) covalently bound through a disulfide bond to the condensing carrier, polyamidoamine dendrimer (Haensler and Szoka, 1993). The GALA peptide was designed both to destabilize lipid bilayers at low pH and to mimic the properties of viral fusogenic proteins (Parente et al., 1990).

In order to increase gene delivery efficiency by effecting plasmid release from the endosomal compartment, we have synthesized short amphipathic peptides (10-20 amino acids) that were added to condensed particles. These peptides were designed to form α-helices at low pH, by protonation of the glutamic acid residues, to expose a hydrophobic face comprised of only strongly apolar amino acids and an hydrophilic face mainly dominated by the glutamic acid residues. This structural conformation favors the partitioning of the amphipathic peptides, or potential clusters of these, into the endosomal membrane, thus effecting the release of the endosomal compartment into the cytoplasm. At physiological pH, the negative charge of the glutamic acid residues maintain the peptides in

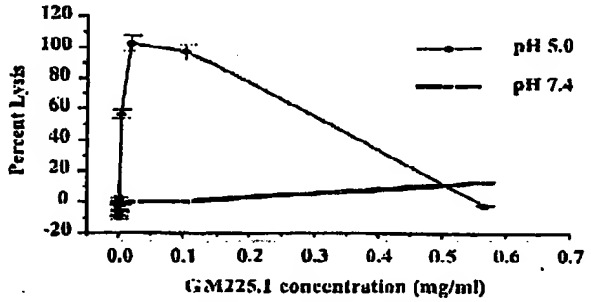

Fig. 4. Hemolytic activity of the GM225.1 peptide at physiological pH (7.4) and endosomal pH (5.0) expressed as percent erythrocyte lysis after one hour based on 100% for Triton X100.

a random coil conformation, preventing them from destabilizing biological membranes. The activity of these novel fusogenic peptides on biological membranes has been shown to be pH-dependent in an hemolytic assay (Figure 4), the erythrocyte hemolysis only occurring at acidic pH and not at physiological pH (G ttschalk et al., 1996; Tomlinson and Rolland, 1996).

Net positively charged peptide-based complexes, without fusogenic peptides, that interact in a non-specific manner with plasma membranes have a relatively low in vitro cell transfection effici ncy. It has been shown that a positively charged peptide/plasmid complex, without any fusogenic peptide, is unable to transfect efficiently several cell lines in vitro (Duguid et al., unpublished data). However, the addition of chloroquine to the culture medium increased the expression of a reporter gene (β-galactosidase) proportionally to the concentration of chloroquine. The addition of a pH-sensitive lytic peptide to condensed plasmids induced a significantly higher level of expression as compared to that found using the optimal chloroquine concentration (Rolland and Tomlinson, 1996). At a given charge ratio of condensing peptide to DNA plasmid, the transfection efficiency has been shown to be proportional to the concentration of the fusogenic peptide introduced in the complex (Figure 5). The same phenomenon has been observed for glycopeptide-based systems for receptor-mediated gene delivery (Mumper and Wadhwa, personal communication). The addition of amphipathic, pH-sensitive peptides to condensed plasmids has been shown to result in significant increase in transfection efficiency in a number of cell lines in vitro, demonstrating the need for the incorporation of a fusogenic peptide in such peptide-based gene delivery systems.

Combinations of an amphipathic peptide with a condensing peptide have been shown to result in transfection of over 20 different types of cultured cells, including HepG2 cells (Gottschalk et al., 1996). This peptide-based gene delivery system is a promising approach to efficient in vivo gene transfer. The use of these peptides, even without a cell-specific targeting ligand, results in in vitro transfection efficiencies in HepG2 cells similar to those found with adenoviral-mediated gene transfer (Gottschalk et al., 1996). The prototype peptide gene delivery system used for transfecting HepG2 cells has been found to be non-cytotoxic at the concentrations used for in vitro studies, whereas the adenoviral vectors, at a multiplicity of infection (MOI) of 100:1 approach their toxic dose, i.e., a MOI of 1000:1 killed all the HepG2 cells (Gottschalk et al., 1996). This peptide gene delivery system was also found to be more than 1,000-fold less toxic to HepG2 cells in vitro than poly-L-lysine-based systems. Repeated subcutaneous or intraperitoneal injections of the peptide gene delivery system to mice has shown no induction of a humoral response after 90 days (Smith et al., personal communication).

CONCLUDING REMARKS

While the expectations and the promise of gene therapy are high for the future,

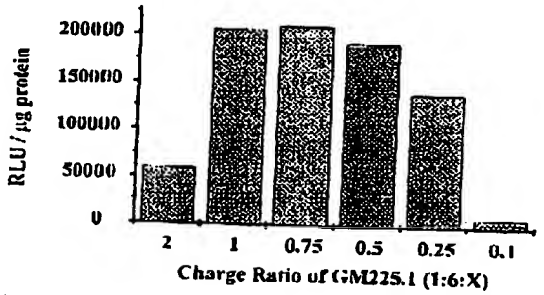

Fig. 5. β-galactosidase expression after 48h in C_2C_{12} myoblasts transfected in 10% serum-containing medium with a plasmid condensed by a cationic peptide (GM208) at a charge ratio of 1:6 (-/+) in presence of different concentrations of a pH-sensitive fusogenic peptide (GM225.1).

clinical efficacy of any gene therapy approach has not been definitively shown to date. The limitations of actual gene therapy methods have to be faced and research to improve on the control of gene location and expression in the patient's body needs to be pursued. Early g ne therapy methods, that relied on the use of viruses or implanted cells t transfer therapeutic genes into the body, have not met, in general, their expectations in terms of safety and clinical efficacy. Today, there is consequently an increasing interest in developing efficient non-viral methods, that can provide for the control of gene location and gene function after in vivo administration to patients. These novel approaches have progressed to the clinic and offer the potential of safe and effective gene therapy. Many advanced drug delivery systems that have been developed during the past several years (Tomlinson, 1987; Rolland, 1993) are having a direct and positive utility in the development of effective non-viral gene delivery systems.

Controllable, non-viral gene therapy holds great promise for providing products that will effectively improve upon the delivery and use of proteins that have poor pharmacokinetic profiles. Gene medicines are designed to provide a safe and cost-effective treatment for a variety of severe and debilitating diseases, as well as to enhance patient compliance as compared to conventional pharmaceutical and biological products. They may also enable unique opportunities in the development of novel products that produce intracellular proteins.

Each biological target will require specific gene medicines to control both location and function of a gene within the patient's body. The successful development of non-viral gene medicines will require a multidisciplinary approach to design advanced synthetic gene delivery systems able to self-assemble with plasmid-based gene expression systems. The ultimate challenge for gene therapy will be to develop products to be used as pharmaceuticals that can gain physician and patient acceptance and compete with conventional therapies by improving on safety, efficacy, compliance and cost.

Acknowledgments

I would like to acknowledge Dr. John Duguid and Mark Logan for obtaining and computing the data presented in Figures 1, 4 and 5 as well as Dr. Frank Tagliaferri for the molecular modelling of the galactosylated peptide interaction with plasmid DNA as shown in Figure 3. I would like to express my gratitute to Dr. Maria Bruno for the analysis presented in Table 2 and Dr. Eric Tomlinson for his valuable insights into the biological and pharmaceutical issues related to gene delivery. The support of many of the scientists at GENEMEDICINE, INC. in generating the data presented in this manuscript is gratefully acknowledged.

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